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Distinctive and pervasive alterations in aqueous humor protein composition following different types of glaucoma surgery

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Purpose: To investigate whether specific glaucoma surgeries are associated with differences in aqueous humor protein concentrations compared to eyes without filters.

Methods: In this cross-sectional study, aqueous humor samples were prospectively collected from control subjects who underwent routine cataract surgery (n=14) and from patients who had different glaucoma filters: Baerveldt aqueous shunt (n=6), Ahmed aqueous shunt (n=6), trabeculectomy (n=5), and Ex-Press trabeculectomy (n=3). Total protein concentrations were determined with Bradford assay. Tryptic digests were analyzed with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Proteins were identified with high confidence using stringent criteria and were quantitatively compared with a label-free platform. Relative protein quantities were compared across groups with ANOVA. Post hoc pair-wise comparisons were adjusted for multiple comparisons.

Results: Compared to the control eyes, the aqueous humor protein concentration was increased approximately tenfold in the Ahmed and Baerveldt eyes and fivefold in the trabeculectomy and Ex-Press eyes. Overall, 718 unique proteins, splice variants, or isoforms were identified. No differences in the protein concentrations were detected between the Baerveldt and Ahmed groups. Likewise, the trabeculectomy and Ex-Press groups were remarkably similar. Therefore, the aqueous shunt groups were pooled, and the trabeculectomy groups were pooled for a three-way comparison with the controls. More than 500 proteins differed significantly in relative abundance (ANOVA $p<0.01$) among the control, aqueous shunt, and trabeculectomy groups. Functional analyses suggested these alterations in relative protein abundance affected dozens of signaling pathways.

Conclusions: Different glaucoma surgical procedures were associated with marked increases in the aqueous humor protein concentration and distinctive changes in the relative abundance of numerous proteins involved in multiple signaling pathways.

Ocular health relies on maintaining a balance between the inflow and outflow of the aqueous humor, the biologic fluid found in the anterior part of the eye. Aqueous humor is produced by the ciliary body and passes from the posterior chamber through the pupil into the anterior chamber, where the aqueous humor drains through the trabecular meshwork out of the eye into the venous system through Schlemm's canal [1]. When aqueous inflow exceeds outflow, it may cause increased intraocular pressure that can damage the optic nerve [1]. Glaucoma is a group of diseases involving optic nerve deterioration because of increased intraocular pressure. Surgical techniques used to treat glaucoma typically rely on increasing aqueous outflow. These techniques include trabeculectomy and aqueous shunt implantation. Medicare claims data and surveys of glaucoma surgeons indicate that aqueous shunts are being increasingly used in the management of glaucoma [2].

Patients with a history of glaucoma surgery have an increased risk of cornea endothelial decompensation and corneal transplant failure [3-10]. We hypothesized that glaucoma surgery causes changes in the protein milieu of aqueous humor that may adversely affect corneal endothelial survival. Previously, we found evidence of aqueous humor alterations in patients who had undergone aqueous shunt surgery [11]. In this follow-up study, we sampled a new set of patients and used more advanced analytical techniques that were able to detect far more proteins. We collected aqueous humor from eyes that had undergone four types of glaucoma surgery: Baerveldt aqueous shunt (non-valved device, Advanced Medical Optics, Santa Ana, CA), Ahmed aqueous shunt (valved device, New World Medical, Inc., Rancho Cucamonga, CA), trabeculectomy, and Ex-Press implantation in conjunction with a trabeculectomy (Optonol, Ltd., Neve Ilan, Israel). These samples were compared with control samples obtained from patients scheduled for routine cataract surgery.

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METHODS

This cross-sectional study involved the collection of 37 aqueous humor samples from 37 patients between 21 October 2011 and 5 March 2014 at Price Vision Group (Indianapolis, IN). Study standards conformed to the ethical guidelines set forth in the Declaration of Helsinki. Permission was obtained from an independent review board (IRB) to conduct the study. The study was explained to patients, and signed informed consent was obtained before sampling.

Collection of human aqueous humor: Samples were drawn from only one eye per patient. To minimize risk to participants, the samples were usually drawn during a scheduled intraocular surgery. Topical proparacaine, an anesthetic, was applied to the patient's eye. A 30-gauge needle was used to pierce the corneal edge and suction 0.1 to 0.2 ml of anterior chamber fluid. Two samples were discarded immediately by the surgeon because of suspected contamination with blood or iris particles. The remaining samples were stored in liquid nitrogen until analysis.

Sample preparation: The protein concentration for each aqueous humor sample was determined by the Bradford assay [12]. Human aqueous humor contains several high abundance plasma proteins, so immune depletion was performed to improve the identification of less abundant proteins. The samples were separated into bound and flow-through (depleted) fractions using Pierce Top 2 Abundant Protein Depletion Spin Columns (Thermo Scientific, Waltham, MA, Product #85162). The fractionation was performed according to the manufacturer's instructions. The albumin- and immunoglobulin-depleted aqueous humor protein samples were reduced, alkylated, and tryptically digested using a conventional method previously published by Lai et al. [13].

LC-MS/MS: Sample digests were analyzed using a Thermo Scientific Orbitrap Velos Pro Hybrid Ion Trap-Orbitrap Mass Spectrometer coupled with a Surveyor autosampler and mass spectrometry (MS) high-performance liquid chromatography (HPLC) system (Thermo Scientific). Tryptic peptides were injected onto a C18 reversed phase column (TSKgel ODS-100V, 3 µm, 1.00 mm × 150 mm) at a flow rate of 50 µl/min. The mobile phases A and B were liquid chromatography-mass spectrometry (LC-MS) grade H_2O with 0.1% formic acid and acetonitrile with 0.1% formic acid, respectively. The gradient elution profile was as follows: 3% B for 8 min, 10–35% B for 92 min, 35–80% B for 12 min, and 80% B for 8 min. The data were collected in the Data dependent MS/MS mode of Fourier transform-ion trap (MS-MS/MS) with the electrospray ionization interface using normalized collision energy of 35% (collision induced dissociation). Dynamic exclusion settings were set to repeat count=1, repeat duration=30 s, exclusion duration=45 s, and exclusion mass width=10 ppm (low) and 10 ppm (high).

Protein identification and quantification: The acquired data were searched against the UniProt protein sequence database of HUMAN (released on 19 March 2014) using X!Tandem algorithms in the Trans-Proteomic Pipeline (TPP, v. 4.6.3). General parameters were set to the following: parent monoisotopic mass error set as 10 ppm, cleavage semi set as yes, missed cleavage sites set at 2, and static modification set as + 44.026215 Da on Cysteine. The searched peptides and proteins were validated with PeptideProphet and ProteinProphet in TPP [14,15]. Only proteins and peptides with protein probability ≥0.9000 and peptide probability ≥0.8000 were reported. Protein quantification was performed using a label-free quantification software package, IdentiQuantXL [16].

To assess for possible sample contamination with blood during the aqueous tap, the hemoglobin levels were compared across samples. One control sample was an outlier, suggesting that it may have been contaminated with blood, so the data from that sample were excluded from the analysis.

Pathway analysis: Qiagen's Ingenuity Pathway Analysis (IPA®, Qiagen, Redwood City, CA) was used to assess the potential functional implications of altered protein abundances. An activation z-score of ±2 was considered significant.

Statistical methods: The false discovery rate (FDR) [16] was estimated using Q-value software. Critical F-ratio significance for ANOVA was set at $p<0.01$ corresponding to $q<0.003$ or a false discovery rate of only 0.3%.

One-way ANOVA and pairwise multiple comparisons (the Holm-Sidak method) [17] were used to assess mean protein abundance differences across the groups studied. Tests were two-sided, and p values less than 0.01 were considered statistically significant.

RESULTS

Demographics: Table 1 lists the demographics of the 34 subjects (34 samples) included in the comparative analysis (three samples were not included because of the suspected contamination described above). The 14 subjects in the control group had not had previous intraocular surgery, and the aqueous sample was collected at the time of the scheduled cataract extraction.

The subjects in the glaucoma surgery groups had previous cataract extraction as well as the respective glaucoma procedure. One of three eyes in the Ex-Press group and one of five eyes in the trabeculectomy group had the indicated

procedure twice. In the Ahmed group, one of six eyes had the procedure twice, and three eyes had a prior non-functioning trabeculectomy. In the Baerveldt group, one of six eyes had the procedure twice, and two eyes had prior non-functioning trabeculectomies. The mean (range) time interval between the aqueous collection and the last glaucoma surgery was 2 years (1 to 4 years) in the Ex-Press group, 12 years (2 to 20 years) in the trabeculectomy group, 2 years (2 months to 4 years) in the Ahmed group, and 10 years (2.5 to 21 years) in the Baerveldt group.

Total protein concentration: The total protein concentrations in aqueous humor differed significantly between the groups (p=0.004); see Figure 1. Post hoc pairwise comparisons showed that the control group differed significantly from both aqueous shunt groups (p<0.01); the median protein content was an order of magnitude higher in the Ahmed and Baerveldt groups (0.91 mg/ml and 1.25 mg/ml, respectively) compared with the control group (0.09 mg/ml). The median protein content in the Ex-Press shunt and trabeculectomy groups (0.54 mg/ml and 0.44 mg/ml, respectively) was five- to sixfold higher than that in the control group (0.09 mg/ml), although the differences did not reach the level of statistical significance.

Protein identification and relative concentrations: Mass spectrometry revealed a total of 5,574 peptides, representing 718 unique proteins, splice variants, or isoforms that were identified with high confidence using stringent criteria (Appendix 1 and Appendix 2 – Protein Quantitation and Statistics, worksheet entitled "Proteins sorted by Name"). The relative abundance of the different proteins varied over a range spanning seven orders of magnitude.

We compared the fold-difference in the quantities of each identified protein between groups to determine patterns of protein prevalence. The Ahmed and Baerveldt groups had remarkably similar protein abundance profiles with no significant differences in the relative concentrations of any of the 718 identified proteins/isoforms (Figure 2A). Likewise, the protein abundance profiles were highly similar in the Ex-Press and trabeculectomy groups; only one of the 718 proteins/isoforms differed by a statistically significant amount (Figure 2B).

Given the lack of significant differences between eyes that had undergone similar glaucoma procedures, the Baerveldt and Ahmed groups were pooled as an aqueous shunt group, and the Ex-Press and trabeculectomy groups were pooled as a trabeculectomy group for a three-way comparison with the control group. The relative abundance of 514 of the 718 proteins (72%) differed significantly among the three groups (Supplemental Data 1 – Protein Quantitation and Statistics, worksheet entitled "ANOVA <0.01," sorted by p value from lowest to highest). Nearly one third of the identified proteins (203 of 718) differed significantly in relative abundance between the control and aqueous shunt groups (Figure 2C), and more than half (380 of 718) differed significantly between the control and trabeculectomy groups (Figure 2D). The different types of glaucoma surgical procedures altered relative protein abundance in distinctive ways with more than one third of the identified proteins (256 of 718) differing significantly between the aqueous shunt and trabeculectomy groups (Appendix 1 – Protein Quantitation and Statistics, worksheet entitled "ANOVA <0.01").

IPA functional analyses suggested that the pervasive alterations in relative protein abundance detected in the eyes that had undergone glaucoma surgery likely affected dozens of aqueous signaling pathways with distinctive differences after aqueous shunt versus trabeculectomy procedures (Appendix 3, Appendix 4, Appendix 5, and Appendix 6 – Functional Analyses Controls versus Aqueous Shunts and

Table 1. Subject demographics.

Group	Subjects (number)	Age, years median (range)	Sex, number female:male	Scheduled surgery at time of aqueous draw
Control	14	66 (49–73)	7:7	15 cataract extraction
Ex-Press	3	82 (70–96)	2:1	3 Initial cornea transplant
Trabeculectomy	5	78 (66–94)	4:1	4 Initial cornea transplant 1 Repeat cornea transplant
Ahmed	6	75 (57–88)	3:3	2 Initial cornea transplant 2 Repeat cornea transplant 1 Pars planna vitrectomy 1 Placement of another shunt
Baerveldt	6	62 (55–80)	2:4	3 Initial cornea transplant 1 Repeat cornea transplant 2 Drawn during office exam

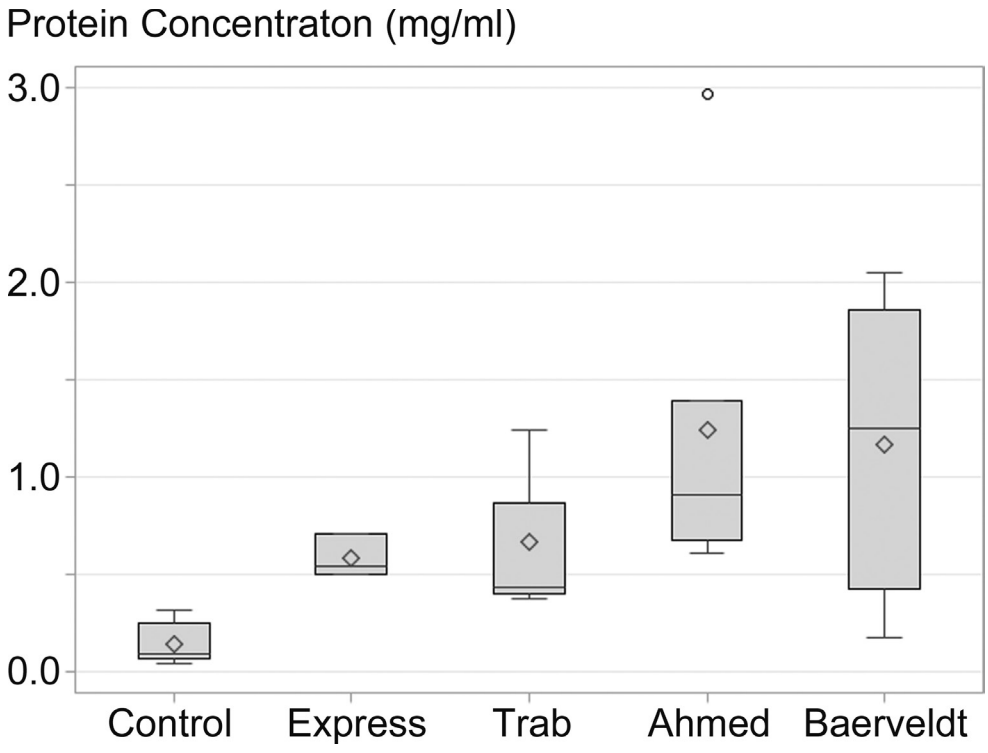

Figure 1. Total protein concentration in aqueous humor by group. In the box plots, the horizontal lines denote medians, the bottom and top of the boxes represent the 25th and 75th percentiles, respectively, and the vertical lines extend to the minimum and maximum values.

Appendix 7, Appendix 8, Appendix 9, and Appendix 10 – Functional Analyses Controls versus Trabeculectomies).

DISCUSSION

This study had three key findings. First, the total protein abundance levels were substantially increased in eyes that had undergone glaucoma surgery. Aqueous shunt implantation was associated with an order of magnitude increase in the overall aqueous protein concentration, while trabeculectomy was associated with approximately a fivefold increase. Second, the relative quantities of numerous proteins were altered in distinctive ways depending upon the type of glaucoma procedure performed. Third, IPA functional analysis suggested the altered protein concentrations impact numerous signaling pathways. Together, these findings may help explain why glaucoma filtering surgeries are associated with increased corneal decompensation from endothelial cell failure in virgin corneas and after corneal transplantation [3-10,18].

Using stringent criteria, we identified 718 unique proteins, isoforms, or splice variants in aqueous humor, contributing to further characterization of this key ocular milieu. With our stringent criteria and low false discovery rate, we confirmed 80% of the 206 proteins identified in triplicate groups in an earlier human proteome analysis by Chowdhury et al. [19]

Singular patterns of protein quantities existed within the different glaucoma surgery groups. The Ahmed and Baerveldt groups were remarkably similar and thus can be considered to have their own "aqueous fingerprint." Similarly, the trabeculectomy and Ex-Press groups had their own "aqueous fingerprint." These signature differences suggest that specific types of glaucoma surgical procedures cause distinctive changes in aqueous humor.

The Ex-Press is a small, stainless steel implant that functions as a modification of a traditional trabeculectomy by replacing the sclerostomy step. Both procedures allow egress of aqueous fluid under a scleral flap. The strong correlation in the total protein abundance, as well as the relative quantities of individual proteins in the Ex-Press and trabeculectomy groups, is consistent with the overall similarity of the two procedures.

In contrast, an aqueous shunt consists of a silicone flow tube that is inserted into the anterior chamber or posterior chamber and connecting silicone plate(s) that are implanted underneath the conjunctiva and Tenon's capsule to create a bleb. The tube allows the flow of aqueous fluid out of the eye. Non-valved aqueous shunts, such as the Baerveldt, sometimes require the ligation of the tube until the newly formed bleb is

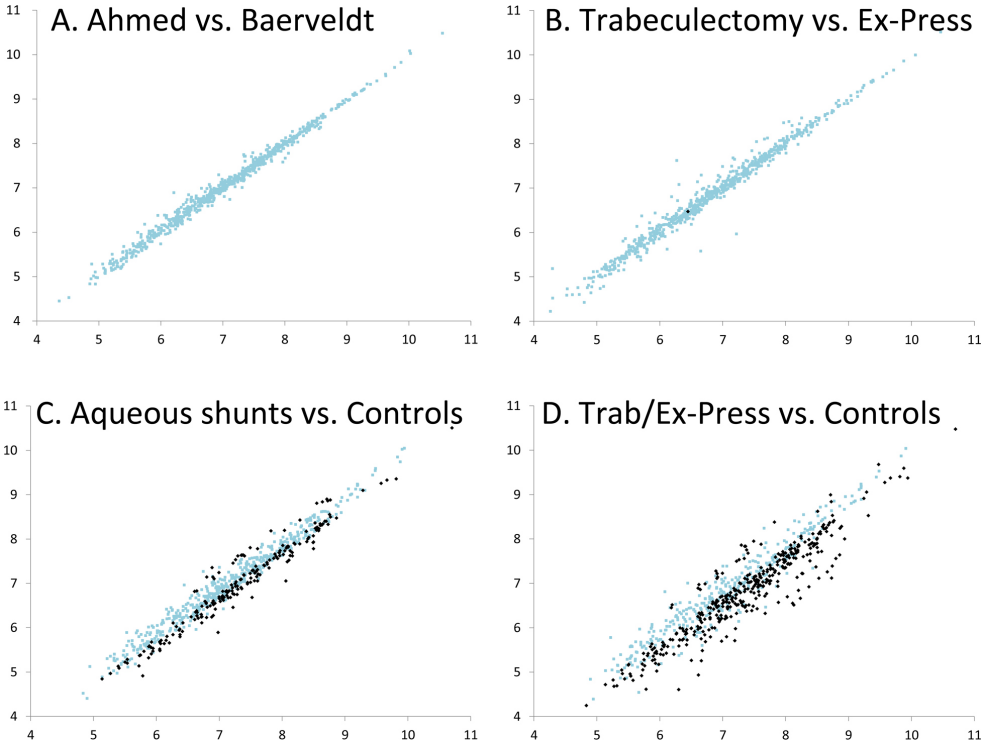

Figure 2. Scatter plots comparing the relative abundance of 718 proteins/isoforms between specified groups were plotted on a log-log scale spanning seven orders of magnitude. The black points represent proteins whose abundance differed significantly between the groups while the aqua points represent proteins whose abundance did not differ significantly between the groups. In some cases, the relative abundance of a given protein differed substantially between the groups, but the difference was not statistically significant because the abundance also varied considerably among the individuals within each group. **A**: No proteins differed significantly in relative abundance between the Ahmed and Baerveldt groups. **B**: Only one of the 718 proteins differed significantly between the trabeculectomy and Ex-Press groups. **C**: The relative abundance of 203 proteins differed significantly between the control group (x-axis) and the pooled aqueous shunt groups (Ahmed and Baerveldt, y-axis). **D**: The relative abundance of 380 proteins differed significantly between the control group (x-axis) and the pooled trabeculectomy and Ex-Press groups (y-axis).

mildly fibrosed and watertight to reduce the risk of hypotony, or insufficient intraocular pressure, in the early postoperative period. Valved implants, such as the Ahmed, attempt to reduce the risk of postoperative hypotony with a mechanical valve. The similarity in the relative protein quantities in the Ahmed and Baerveldt groups suggests that the valve intended to prevent hypotony and fluid backflow into the eye with the Ahmed shunt did not significantly influence the substantial alteration in aqueous composition caused by the presence of the aqueous shunt.

Corneal decompensation is a known risk factor after glaucoma surgery. In a tube versus trabeculectomy (TVT) study, persistent corneal edema lasting more than a month was observed in 6% of the trabeculectomy eyes and in 9% of the aqueous shunt eyes [3]. Furthermore, 3% of the eyes in both groups required corneal transplantation within a 3-year follow-up period [3]. In the Ahmed Baerveldt Comparison Study, the incidence of persistent corneal edema was 6.4% in the Ahmed group and 10.1% in the Baerveldt group, after patients with preexisting corneal disease as well as those who experienced early postoperative edema that subsequently resolved were excluded [18]. A prospective evaluation of corneal endothelial cell loss within the first 2 years after Ahmed aqueous shunt implantation found increasing cell loss over time: 12% loss at 6 months, 15% at 12 months, and 19% at 24 months [10].

Multiple studies have shown that prior glaucoma surgery is a highly significant risk factor for endothelial cell decompensation in corneal grafts [4-9]. In a study of 453 endothelial keratoplasty procedures, we found that prior glaucoma surgery was the single most important risk factor for graft failure [7]. The 5-year survival rate for endothelial grafts in that study was only 25% in eyes with a prior aqueous shunt and 59% for eyes with a prior trabeculectomy, compared with 95% in eyes without a glaucoma filter [7]. Prior aqueous shunt surgery increased the relative risk of graft failure within 5 years by 3.6-fold while trabeculectomy increased the risk of failure by 1.5-fold [7]. Similarly, in a series of 77 penetrating keratoplasty eyes with prior Ahmed valves, Hollander et al. found graft survival was only 58% at 1 year and 41% at 3 years [8]. Potential confounding factors were that the eyes in those studies may have been on different glaucoma medications, undergone multiple surgeries, or had poorly controlled intraocular pressure.

Overall, the corneal endothelium in a grafted eye appears to be less resilient than a virgin cornea without a graft, and

grafted eyes may be an indicator of what happens to virgin non-grafted corneas over a longer timeframe. Therefore, it is important to develop a better understanding of why grafts and virgin corneas experience endothelial failure after glaucoma surgery and to find treatments to protect the endothelium.

Aqueous shunts extend into the anterior chamber, where the tube can come into contact with the corneal endothelium if the tube is not properly trimmed and positioned. Direct contact between the tube and the corneal endothelium is often thought to be a cause of corneal endothelial decompensation. However, the risk of corneal endothelial decompensation is increased even when the tube is positioned in the pars plana well away from the cornea [9], suggesting that aqueous shunt implantation fundamentally changes the ocular environment in a way that is deleterious to the corneal endothelium. Avascular tissues, such as the cornea, receive external cues from components of the aqueous humor. In particular, since corneal endothelial cells are generally in a quiescent state and not regenerating in adult humans, the cells may be particularly sensitive to the appropriate balance of regulatory proteins within the aqueous humor.

Among the strengths of this study were the conservative criteria we used for identifying proteins as well as for determining which proteins differed significantly in relative quantities between the different groups. Furthermore, the aqueous samples were analyzed individually, rather than pooled; this allowed us to take biologic variations between individuals into consideration. An additional strength was the sampling from two independent aqueous shunt groups, which validated each other, as well as the sampling of the trabeculectomy and Ex-Press groups, which likewise validated each other.

A study limitation is that it would have been useful to have a control group of patients with primary open angle glaucoma (POAG) who had not yet undergone glaucoma surgery, because inadequate medical control of POAG is the most common reason for trabeculectomy or aqueous shunt surgery. However, Izzotti et al. showed that the total protein abundance in aqueous humor was not significantly elevated in eyes with primary open angle glaucoma [20]. This supports the concept that the glaucoma surgery, not the underlying glaucoma, caused the marked increase in aqueous protein abundance that we detected. This is consistent with findings that 5-year endothelial keratoplasty survival in eyes with medically-managed glaucoma (no prior glaucoma surgery) was comparable to that in control eyes without glaucoma [7].

Another study limitation was the lack of historical data on intraocular pressure control and ocular inflammatory reactions in the glaucoma surgery groups. In addition, it would have been useful to have a control group that had undergone cataract surgery alone and to have sampled eyes with prior glaucoma surgery that had not experienced corneal decompensation, but concern about minimizing risk for participants by scheduling the aqueous sampling in conjunction with a planned surgical procedure imposed limitations. Given these limitations, it was fascinating to find that the relative concentrations of hundreds of proteins differed between the aqueous shunt and trabeculectomy groups. Thus, these two types of glaucoma procedures each were associated with a distinctive protein fingerprint, despite aspects the two groups had in common, including prior cataract surgery and the prevalence of corneal decompensation.

In conclusion, we have expanded the number of proteins identified in aqueous humor to date and shown that aqueous shunts and trabeculectomies significantly alter the balance of protein abundance levels in the aqueous humor in distinctive ways. The precise etiology of corneal endothelial decompensation and graft failure in eyes with prior glaucoma surgery requires further elucidation. This study helps clarify how different types of glaucoma procedures impact the microenvironment of corneal endothelial cells and will hopefully contribute to new hypotheses and approaches to help prevent corneal decompensation following glaucoma surgery.

APPENDIX 1. PROTEIN QUANTIFICATION AND STATISTICS

To access the data, click or select the words "Appendix 1."

APPENDIX 2. PROTEIN QUANTIFICATION AND STATISTICS FOR PROTEINS THAT DIFFERED SIGNIFICANTLY BETWEEN GROUPS

To access the data, click or select the words "Appendix 2."

APPENDIX 3. FUNCTIONAL ANALYSIS OF AQUEOUS SHUNTS VERSUS CONTROLS CANONICAL PATHYWAYS

To access the data, click or select the words "Appendix 3."

APPENDIX 4. FUNCTIONAL ANALYSES AQUEOUS SHUNTS VERSUS CONTROLS UPSTREAM REGULATORS

To access the data, click or select the words "Appendix 4."

APPENDIX 5. FUNCTIONAL ANALYSES AQUEOUS SHUNTS VERSUS CONTROLS BIOFUNCTIONS

To access the data, click or select the words "Appendix 5."

APPENDIX 6. FUNCTIONAL ANALYSES AQUEOUS SHUNTS VERSUS CONTROLS NETWORKS

To access the data, click or select the words "Appendix 6."

APPENDIX 7. FUNCTIONAL ANALYSES TRAB GROUP VERSUS CONTROLS CANONICAL PATHWAYS

To access the data, click or select the words "Appendix 7."

APPENDIX 8. FUNCTIONAL ANALYSIS TRAB GROUP VERSUS CONTROLS UPSTREAM REGULATORS

To access the data, click or select the words "Appendix 8."

APPENDIX 9. FUNCTIONAL ANALYSIS TRAB GROUP VERSUS CONTROLS BIOFUNCTIONS

To access the data, click or select the words "Appendix 9."

APPENDIX 10. FUNCTIONAL ANALYSIS OF TRAB GROUP VERSUS CONTROLS NETWORKS

To access the data, click or select the words "Appendix 10."

ACKNOWLEDGMENTS

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